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# The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation

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## Abstract

**Aim:** To determine the presence and location (stroma versus epithelium) of citrullinated proteins in periodontitis tissue as compared to non-periodontitis tissue and synovial tissue of RA patients.

**Materials & Methods:** Periodontitis, healthy periodontal and RA-affected synovial tissue samples were collected in addition to buccal swabs. These samples were stained for the presence of citrullinated proteins using polyclonal (Ab5612) and monoclonal (F95) antibodies. Furthermore, Western blotting with F95 was performed on lysates prepared from periodontal and synovial tissues.

**Results:** In periodontitis stroma, increased citrullinated protein presence (80%) was observed compared with control stroma (33%), the latter was associated with inflammation of non-periodontitis origin. Periodontal epithelium always stained positive for Ab5612. Noteworthy, only periodontitis-affected epithelium stained positive for F95. All buccal mucosal swabs and 3 of 4 synovial tissue samples stained positive for both Ab5612 and F95. Western blotting with F95 showed presence of similar citrullinated proteins in both periodontitis and RA-affected synovial tissue.

**Conclusion:** Within the periodontal stroma, citrullination is an inflammation-dependent process. In periodontal epithelium, citrullination is a physiological process. Additional citrullinated proteins are formed in periodontitis, apparently similar to those formed in RA-affected synovial tissue. Periodontitis induced citrullination may play a role in the aetiology of rheumatoid arthritis.

Key words: ACPA; citrullinated proteins; periodontitis; rheumatoid arthritis

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Hippocrates was probably the first (400 BCE) to report on the association between oral health and 'rheumatism' by reporting a case of joint pain being successfully treated after extraction of a single tooth

(O'Reilly & Claffey 2000). More recently, evidence from epidemiological studies has pointed out that patients with rheumatoid arthritis (RA) have a significantly increased prevalence of periodontitis (Kasser et al. 1997, Deodhar et al. 1998, Gleissner et al. 1998, 2003, Abou-Raya et al. 2005, 2008, Biyikoglu et al. 2006, Pischon et al. 2008, de Pablo et al. 2009). Moreover, disease severity of the one (RA or periodontitis) seems to be related to disease severity of the other (Mercado et al. 2001, Marotte et al. 2006, Nilsson & Kopp 2008). To prove that Hippocrates' statement might not have been far from the truth, recent intervention studies pointed towards a beneficial effect of treating periodontitis on RA disease activity; i.e. a reduction in number of tender and swollen joints, CRP, ESR, VAS-scores, and consequently DAS28, was observed after treating periodontitis (Ribeiro et al. 2005, Al-Katma et al. 2007, Ortiz et al. 2009).

Anti-citrullinated protein antibodies (ACPA) are highly specific for RA and are suspected of playing a role in the aetiology of RA (Avouac et al. 2006, Nishimura et al. 2007, Klareskog et al. 2008). The discovery of ACPA in 1964, then called anti-perinuclear factor, was made on buccal epithelial cells (Nienhuis & Mandema 1964). In addition to the presence of citrullinated proteins in buccal epithelium from within the oral cavity, the periodontal epithelium may also contain citrullinated proteins. In fact, periodontitis has been hypothesized as being an inducer of anti-citrullinated protein antibody (ACPA) formation (Rosenstein et al. 2004). In support of this hypothesis, the presence of periodontitis in RA has recently been shown to be associated with ACPA positivity (Dissick et al. 2010). There are further lines of evidence in support of periodontitis as inducer of ACPA formation. First, both periodontitis and ACPA formation share the same environmental and genetic risk factors, viz. smoking (Nunn 2003, Sugiyama et al. 2010) and HLA-DR4 shared epitope (Bonfil et al. 1999). Second, *Porphyromonas gingivalis*, a major pathogen in periodontitis, is the only known bacterium to contain a pept-

idyl arginine deiminase (PAD) enzyme (Wegner et al. 2010). The formation of citrullinated proteins is catalysed by PAD enzymes. Therefore, PAD formed by *P. gingivalis* (PPAD) may cause citrullinated protein formation, which may in turn elicit an antibody response to these proteins, i.e. lead to ACPA formation. Furthermore, citrullinated  $\alpha$ -enolase peptide 1, is considered to be a major auto-antigen in RA. This immunodominant peptide showed 82% homology with citrullinated enolase from *P. gingivalis*. Thus, ACPA response to synovial citrullinated  $\alpha$ -enolase may be induced by *P. gingivalis* produced citrullinated enolase in the periodontium (Lundberg et al. 2008). Moreover, antibody titres to *P. gingivalis* are correlated with ACPA titres of RA patients (Mikuls et al. 2009). This implies that *P. gingivalis*, either through PAD, citrullinated enolase or otherwise, contributes to inducing ACPA formation. A third line of evidence implicating periodontitis as an inducer of ACPA formation, stems from the fact that auto-antibody formation commonly occurs in periodontitis. Strikingly, the auto-antibodies produced in periodontitis mainly target collagen (Berglundh & Donati 2005). Furthermore, citrullination is known for its ability to break immunological tolerance (Lundberg et al. 2005), and ACPAs are known for recognizing two or more different citrullinated peptides (van de Stadt et al. 2010). Therefore, an auto-antibody (ACPA) response against citrullinated collagen initiated in the periodontium, might cross react with citrullinated collagen in the synovium.

In summary, ACPAs are thought to play an important role in the aetiology of RA and periodontitis and might, for more than one good reason, be involved in the initiation of ACPA formation (Fig. 1). This study was designed to assess the presence and localization (stromal versus epithelial) of citrullinated proteins in periodontitis tissue compared with non-periodontitis tissue. Although auto-antibody formation is known to occur in periodontitis (Berglundh & Donati 2005), the presence of citrullinated proteins is a pre-requisite for the formation of ACPA.

## Material and Methods

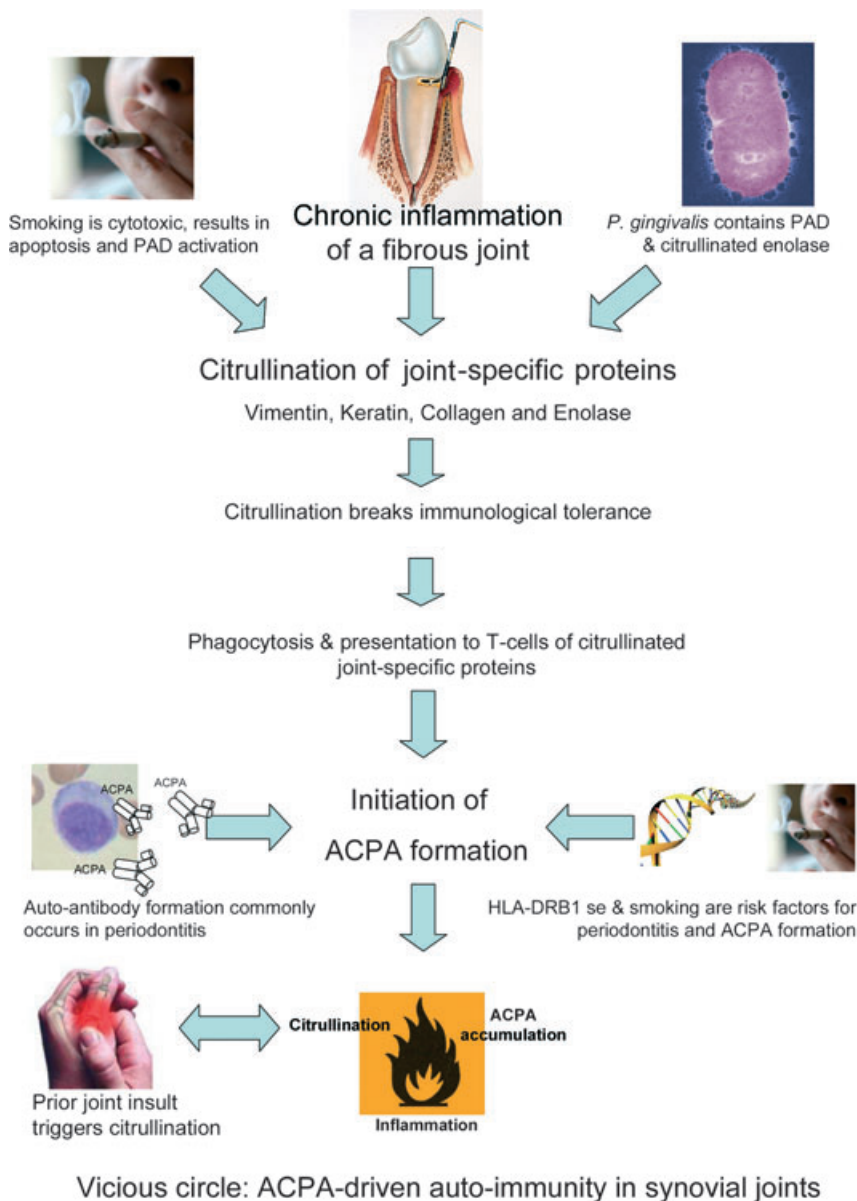
### Patients and tissue samples

Patients with chronic persistent periodontitis, not responding to initial therapy, are routinely subjected to surgical excision of the inflamed part of their periodontium. Periodontal tissue samples of 15 randomly selected periodontitis patients, without any other known medical condition (including RA), undergoing such surgical excision were obtained. Information on age, gender, smoking (pack years) and periodontitis severity was obtained from patients' dental records, in which these data were routinely recorded (Table 1). Clinically non-inflamed periodontal tissue samples (no bleeding on probing, no periodontal pockets  $\geq 4$  mm) were obtained from six patients undergoing prophylactic removal of impacted third molars (wisdom teeth). Synovial tissue samples of four RA patients, previously obtained during joint replacing surgery, and buccal mucosal cell swabs of three healthy donors known to be positive for anti-perinuclear factor staining, were used as positive control tissues for citrullinated protein staining. Informed consent was obtained from all patients and the institutional review board approved this study.

For Western blotting, periodontal and synovial tissue samples of an additional five, randomly selected, periodontitis patients without any other known diseases, three RA patients, and one RA patient with periodontitis, were used. These patients were not the same as those who provided the tissue samples used for immunohistochemistry.

### Processing the tissue samples

After excision, periodontal tissue samples of periodontitis patients were symmetrically dissected. Immediately thereafter, half of the sample was frozen using liquid nitrogen, the other half was put in formaldehyde and later embedded in paraffin. Cryostat sections were fixed in acetone and endogenous peroxidase was blocked with 1% hydrogen peroxide ( $H_2O_2$ ) in phosphate buffered saline (PBS). Then cryostat sections were incubated with either of two primary



**Fig. 1.** Hypothetical model: periodontitis initiates ACPA formation. Periodontitis, together with exposure to tobacco smoke and PAD enzymes from *P.gingivalis* (a major pathogen causing periodontitis) may cause citrullination of joint-specific proteins, i.e. auto-antigens implicated in RA. In conjunction with the HLA-DRB1 shared epitope and exposure to tobacco smoke, an ACPA response could be initiated in the periodontium. ACPAs, anti-citrullinated protein antibodies; HLA-DRB1 se, human leucocyte antigen complex encoding for the peptide binding pocket of the DRB1 molecule; PAD, peptidyl arginine deiminase; *P.gingivalis*, *Porphyromonas gingivalis*; RA, rheumatoid arthritis.

antibodies: (1) anti-citrullinated protein rabbit polyclonal antibody (Ab5612; Millipore, MA, USA) diluted 1:100 in PBS + 1% bovine serum albumin (BSA), and (2) mouse anti-citrullinated protein IgM monoclonal antibody (F95; kindly provided by A.P.Nicholas, University of Alabama, Birmingham, AL,

USA) diluted 1:1000 in PBS. These two antibodies were selected for their suitability to be used in immunohistochemistry and are the only two currently available antibodies appropriate for immunohistochemical staining. It is possible that F95, which is raised against a deca-citrullinated peptide, only reacts when

multiple citrulline residues are present, whereas Ab5612 can also react with single citrulline residues, as this antibody is raised against citrullin-glutaraldehyde BSA.

Prior to blocking endogenous peroxidase with 1%  $H_2O_2$ , the cryostat sections were blocked with 10% pool serum in PBS when F95 was used. Finally, detection of primary antibodies was performed using respectively (1) a goat anti-Rabbit (Dako, Glostrup, Denmark) diluted 1:50 in PBS + 1% BSA for Ab5612 and (2) a goat anti-Mouse IgM-HRP (Southern Biotech, AL, USA) diluted 1:500 in PBS + 1% AB serum for F95, followed by using a DAB kit (K3467, Dako).

Cryostat sections of six clinically non-inflamed periodontal tissue samples and four synovial tissue samples of RA patients were stained for citrullinated proteins in a similar manner as periodontal tissue samples from periodontitis patients. Likewise, the buccal mucosal swabs, fixed in acetone, were stained. All tissue samples were haematoxylin and eosin (HE) stained. As control for false positive staining, all tissues were additionally stained using control primary antibodies [a mouse IgM isotype control for F95 (Southern Biotech, clone 11E10, 1:1000 in PBS) and rabbit IgG control for Ab5612 (Southern Biotech, nr. 0111-01, 1:100 in PBS + 1% BSA)], followed by identical detection procedures. Finally, the presence of citrullinated proteins in periodontitis tissue and synovium was analysed qualitatively, i.e. either present or absent.

To obtain information on cell types involved in the inflammatory periodontitis lesions (Table 1), paraffin sections of periodontitis tissues were stained for CD3 (Monosan, Uden, the Netherlands, clone PS1, 1:20; T-cells), CD20 (Dako, clone L-26, 1:200; B-cells), CD138 (IQ Products, Groningen, the Netherlands, clone B-A38, 1:80; plasma cells), CD68 (Dako, clone KP1, 1:100; macrophages), CD1a (Immunotech, Marseille, France, clone O10, R.T.U.; dendritic cells), CD31 (Dako, clone JC 70A, 1:40; endothelial cells) and toluidine blue (mast cells). Staining with antibodies was performed using standard methods as described by the manufacturers.

Table 1. Citrullinated protein presence (+) versus absence (-) in periodontitis patients and controls

Variable	Periodontitis Ab5612 + n = 8	Periodontitis Ab5612 - n = 7	p-value	Periodontitis F95 + n = 10	Periodontitis F95 - n = 5	p-value	Periodontitis Either + n = 12	Periodontitis Both - n = 3	p-value	Controls Either weak + n = 2	Controls Both - n = 4
Age in years*	48.9 (11.4)	53.6 (17.9)	0.55*	55.0 (12.4)	43.2 (16.3)	0.14*	52.3 (14.0)	46.2 (18.5)	0.53*	28.5 (5.5)*	28 (8.6)*
PPD in mm*	6.6 (1.2)	6.4 (1.2)	0.67*	6.7 (6.2)	6.2 (0.8)	0.50*	6.6 (1.2)	6.0 (0.9)	0.43*	≤ 3 mm	≤ 3 mm
Female sex†	5 (63%)	5 (71%)	0.71†	5 (50%)	5 (100%)	0.053†	7 (58%)	3 (100%)	0.51‡	2 (100%)†	2 (50%)†
Currently smoking†	4 (50%) yes	2 (29%) yes	0.40†	4 (40%)	2 (40%)	1.0†	5 (42%)	1 (33%)	1.0§	1 (50%)†	1 (25%)†
Packyears‡	0 (0-23)	4 (0-23)	0.71‡	10.5 (15.1)	12.6 (18.3)	0.95‡	0 (0-15)	23 (0-40)	0.24‡	Not assessed	
CD3 + cells/field‡	33 (11-45)	14 (8-45)	0.44‡	31 (12-43)	12 (8-51)	0.55‡	31 (7-39)	11.5 (9-68)	0.55‡		
CD20 + cells/field‡	9 (0-34)	0 (0-18)	0.23‡	1 (0-20)	12 (0-26)	0.84‡	7 (0-35)	0 (0-13.5)	0.84‡		
CD138 + cells/field‡	7 (2-27)	24 (5-100)	0.33‡	7 (3-62)	24 (5-66)	0.55‡	7 (3-62)	24 (5-66)	0.55‡		
CD68 + cells/field‡	3 (0-4)	0 (0-2)	0.13‡	2 (0-5)	0 (0-2)	0.12‡	2 (0-4.5)	0 (0-0)	0.12‡		
CD1a+ cells/field‡	3 (0-6)	1 (0-4)	0.38‡	3 (0-5)	0 (0-4)	0.53‡	2.5 (0-5.5)	0 (0-4)	0.53‡		
CD31 + vessels/field‡	10 (9-14)	9 (6-15)	0.41‡	10 (6-14)	8 (6-16)	0.34‡	9 (6.5-13)	5.5 (5.5-22.5)	0.34‡		
TBlue+ cells/field‡	9 (8-13)	6 (4-24)	0.34‡	10 (8-16)	9 (8-12)	0.35‡	10 (8.5-14.5)	9 (6.5-14)	0.35‡		

\*Variables presented as mean (standard deviation), differences tested for significance using Student's *t*-test.

†Variables presented as numbers (percentages), differences tested for significance using Chi-Square test.

‡Variables presented as median (interquartile range), differences tested for significance using Mann-Whitney *U*-test.

§Differences tested for significance using Fisher's Exact Test.

NB No *p*-values were calculated within the control group (participants without periodontitis) because numbers were either identical or very small (1 or 2).**Analysis of the periodontitis tissue samples**

All positive cells were counted in 10 adjacent fields with a 40× objective (magnification 400×) per periodontitis tissue sample, after which medians and inter-quartile ranges of cell numbers were calculated for each cell type.

**Western blotting of periodontal and synovial tissues**

Periodontal and synovial tissues of randomly selected patients were grinded with a pestle in cell lysis buffer from Cell Signalling (Bioké, Leiden, the Netherlands) to which phenyl-methylsulfonyl fluoride (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added. Samples were subsequently ultra sonicated after which they were diluted once in two times SDS buffer (125 mM Tris-HCl (pH 6.8 at 25°C), 4% w/v SDS, 20% glycerol, 100 mM DTT, 0.02% w/v bromophenol blue) and subsequently heated for 5 min. at 95°C. Samples were loaded on a 10% SDS gel and separated by SDS-PAGE. Proteins were transferred onto PVDF membranes using Criterion electrophoresis and blotting systems (Bio-Rad laboratories, Veenendaal, the Netherlands). After blocking, membranes were incubated with F95 (dilution 1:500). Detection was performed using goat anti-mouse IgM labelled with IRD800CW (Rockland, Gilbertsville, PA, USA). Tebu-Bio Membranes were scanned and analysed using an Odyssey IR scanner (Li-Cor, Lincoln, NE, USA) using Odyssey imaging software 3.0.

**Statistical analysis**

To explore factors possibly linked to the formation of citrullinated proteins in the periodontium, periodontitis patients whose periodontal tissue samples did and did not contain citrullinated proteins were compared with respect to age, gender, smoking, periodontitis severity (probing pocket depth) and the amounts of inflammatory cell types, using student *t*-test, Mann-Whitney *U*- and Chi-square tests as appropriate. Significance level  $\alpha$  was set at 0.05 and subsequently corrected with Bonferroni-Holm correction for multiple comparisons.



## Results

In stroma, citrullinated proteins were detected in 53% ( $n = 8$ ), 67% ( $n = 10$ ) and 80% ( $n = 12$ ) of periodontitis tissue samples using, respectively, Ab5612, F95, or either antibody (Fig. 2a–d, Fig. 3a–d). Of six control periodontal tissue samples, two tissue samples were from persons who had complained of pain and swelling in the previous weeks, although not clinically evident at the time of surgery. Citrullinated proteins were detected in stroma of these two controls only (Figs 2e and 3e citrulline negative control tissue, Figs 2f and 3f citrulline positive con-

trol tissue). In one of these two controls this was apparently associated with an inflammatory infiltrate. Thus, citrullinated proteins were detected in stroma of 33% (2 in 6) of control tissue samples.

Stroma of only 3 of 15 periodontitis patients was negative for citrullinated proteins, i.e. neither AB5612 nor F95 stained positive. For these three patients, there were no macrophages (CD68) per visible field (Table 1). In contrast, in citrulline positive stroma ( $n = 12$ ) median number of macrophages per visual field was 2.0 with an interquartile range of 0–4.5 ( $p = 0.011$ ). This difference was not significant after cor-

recting significance level  $\alpha$  with Bonferroni–Holm correction for multiple comparisons, probably due to small numbers. In Fig. 4, pictures are shown of a citrulline negative and a citrulline positive periodontal tissue to visualize inflammation. Percentage of cell counts in citrulline negative and citrulline positive are given in Fig. 4C.

Likewise, there were no statistically significant differences in age, gender, smoking (current and pack years) and periodontitis severity between periodontitis patients whose stroma contained citrullinated proteins (using Ab5612, F95, or either antibody) and those whose periodontium did not.

In epithelium, citrullinated proteins were detected in periodontitis and control tissue samples whenever using Ab5612. In contrast, when using F95, epithelium stained positive in periodontitis tissue samples only. Citrullinated proteins were detected in three and four of four synovial tissue samples of RA patients using Ab5612 or F95 respectively. The buccal mucosal cell swabs were positive for both antibodies (Fig. 5, only F95 shown).

Western blotting with F95 resulted in a variety of similar bands of citrullinated proteins in all periodontal tissues and in RA-affected synovial tissue with a prominent band around 100 kDa (Fig. 6). Testing reactivity of F95 on citrullinated peptides and their native equivalents by ELISA techniques as described by van de Stadt et al., demonstrated that F95 reacted primarily with citrullinated peptides and not with the native peptides (data not shown) (van de Stadt et al. 2011).

## Discussion

To the best of our knowledge, this was the first study on the presence and location (stromal versus epithelial) of citrullinated proteins in periodontal tissue (Nesse et al. 2009) and is the first full article on this issue. Citrullinated protein presence was observed in 80% of periodontitis-affected stroma compared with 33% of control periodontal stroma (33%). In control periodontal stroma, citrullinated protein presence appeared to be associated with inflammation of non-periodontitis

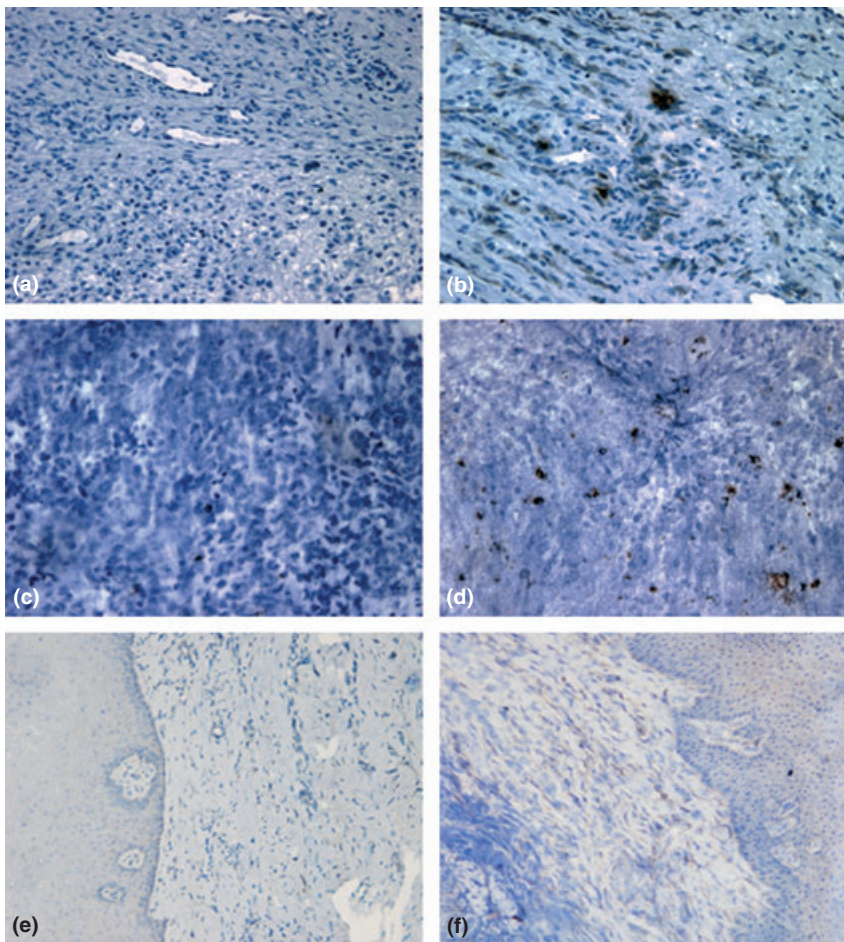


Fig. 2. Citrullinated proteins in periodontium of periodontitis patients, synovium of RA patients and healthy periodontal tissue using rabbit anti-citrullinated protein polyclonal antibody Ab5612, magnification 100 $\times$ . No false positive staining for citrullinated proteins was shown using a rabbit IgG control followed by secondary antibody staining in synovium of RA patient (a) and periodontium of periodontitis patient (c). Brown staining indicates the presence of citrullinated proteins using a rabbit anti-citrullinated protein polyclonal antibody (Ab5612) followed by secondary antibody staining in synovium of RA patient (b) and periodontium of periodontitis patient (d). Healthy periodontal tissue stained negative for citrulline using Ab5612 (e) or positive in the case of presence of inflammation (f).



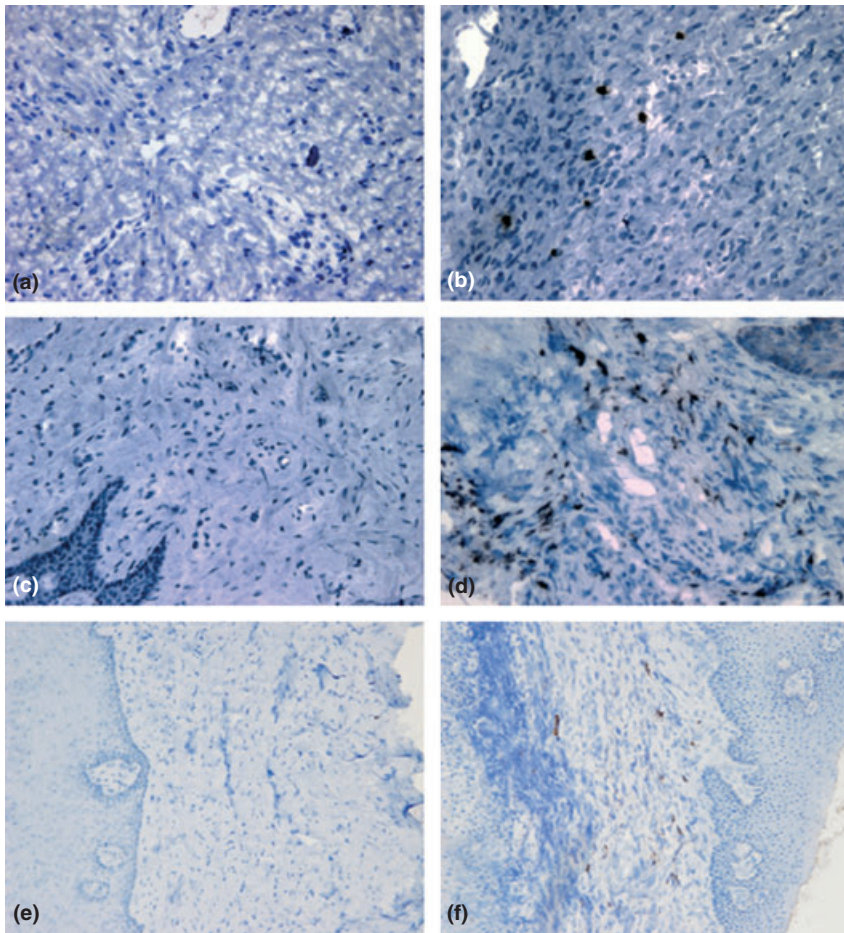


Fig. 3. Citrullinated proteins in periodontium of periodontitis patients, synovium of RA patients and healthy periodontal tissue using mouse anti-citrullinated protein monoclonal antibody F95, magnification 100 $\times$ . No false positive staining for citrullinated proteins was shown using a mouse IgM isotype control followed by secondary antibody staining in synovium of RA patient (a) and periodontium of periodontitis patient (c). Brown staining indicates the presence of citrullinated proteins using mouse anti-citrulline antibody (F95) followed by secondary antibody staining in synovium of RA patient (b) and periodontium of periodontitis patient (d). Healthy periodontal tissue stained negative for citrulline using F95 (e) or positive in the case of presence of inflammation (f).

origin. Recently, our results have been confirmed by others in an abstract, i.e. showing citrullinated protein presence in 80% of periodontitis patients and 40% of healthy controls (Yucel-Lindberg et al. 2010). Thus, citrullinated protein formation within the periodontal stroma appears to be an inflammation-dependent process. This is again in agreement with previous studies showing that the formation of citrullinated proteins in other sites of the human body is inflammation-driven (Makrygiannakis et al. 2006).

In contrast with stroma, periodontal epithelium, whether inflamed or not, always stained positive for

Ab5612. Citrullinated protein detection in periodontal epithelium samples could have been expected. In fact, the discovery of ACPA in 1964, then named anti-perinuclear factor for perinuclear staining pattern, was made on buccal epithelial (i.e. mucosal) cells (Nienhuis & Mandema 1964). Filaggrin, a keratin binding protein abundantly present in epithelium, is known to bind ACPA (Schellekens et al. 1998). Citrullinated proteins have also been shown to be present in the epidermis (Tsuiji et al. 2003). It has even been posed that citrullination plays an essential role in epithelial differentiation through integration and disintegration of ker-

atin filaments. In other words, citrullinated proteins naturally occur in epithelial tissues. However, periodontitis leads to the formation of additional types of citrullinated proteins (targeted by F95) in addition to the types of citrullinated proteins naturally present in non-inflamed periodontal epithelium (targeted by Ab5612 only).

Regarding the different type of citrullinated proteins present in periodontitis tissue compared with healthy periodontal tissue, it is striking that a variety of similar bands of citrullinated proteins (targeted by F95) were found in both synovium of RA and periodontitis tissue, with a strong band around 100 kDa. This may be due to the fact that the periodontium contains many of the cellular and structural components of a joint. The periodontium contains collagens, proteoglycans and hyaluronic acid, major constituents of cartilage (Bartold & Narayanan 2006). Many of the major auto-antigens targeted by ACPA in RA can be found within the periodontium. The periodontal epithelium contains keratin and filaggrin, and periodontal fibroblasts express vimentin. Citrullinated  $\alpha$ -enolase is considered to be another major autoantigen in RA with which *P. gingivalis* produced citrullinated  $\alpha$ -enolases shares, a largely identical immunodominant epitope (Lundberg et al. 2008, 2010, Wegner et al. 2010). The similar bands of citrullinated proteins in periodontitis and RA-affected synovial tissue may point out that similar proteins might get citrullinated in periodontitis and RA-affected synovial tissue. When these identical proteins are degraded by the activity of matrix metalloproteases and subsequently phagocytized by macrophages, as is the case in both RA and periodontitis, an auto-antibody response may be initiated in the periodontium targeting the same components in joints.

In this respect, another striking finding is the fact that the number of macrophages in stroma of periodontitis tissue samples without detectable citrullinated proteins ( $n = 3$ ) is consistently zero, whereas their numbers are small, but almost consistently higher in stroma of periodontitis tissue containing citrullinated proteins. The presence of macrophages thus appears to be associated with citrulli-

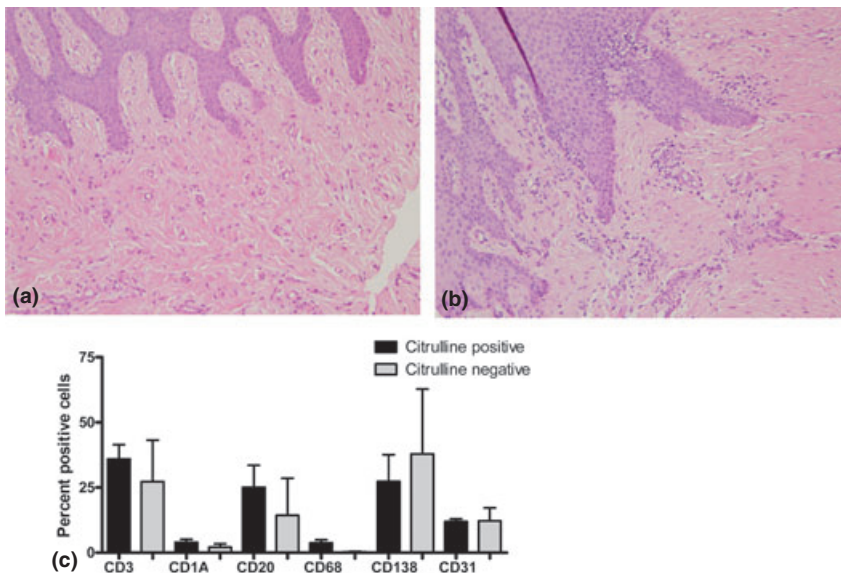


Fig. 4. Inflammation in periodontal tissue of periodontitis patients. (a) Haematoxylin-Eosin (HE) staining of citrulline negative periodontal tissue (magnification 100 $\times$ ). (b) HE staining of citrulline positive periodontal tissue (magnification 100 $\times$ ). (c) Percentage of mean cell numbers of different cell types in citrulline negative and positive periodontal tissues.

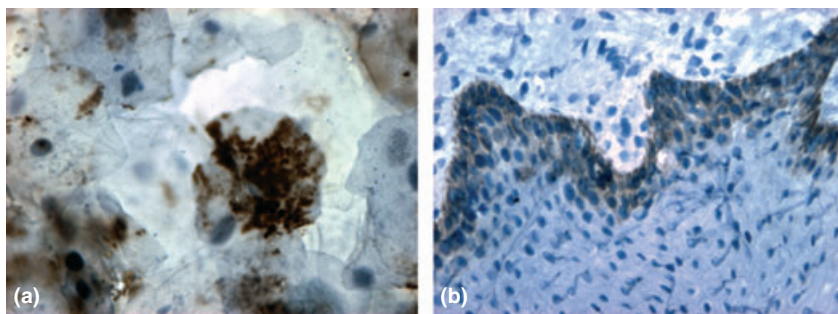


Fig. 5. Citrullinated proteins are present in buccal epithelial cells of healthy subjects and in periodontal epithelium of a periodontitis patient using mouse anti-citrullinated protein monoclonal antibody (F95). (a) Buccal epithelial cells of healthy subjects: The speckled cytoplasmic, perinuclear and nuclear brown staining, as originally described by Nienhuis & Mandema 1964, indicates presence of citrullinated proteins (F95, magnification 400 $\times$ ). (b) Periodontal epithelium of a periodontitis patient: Brown staining indicates presence of citrullinated proteins (F95, magnification 200 $\times$ ).

nated protein formation in periodontitis stroma, and macrophages may play a role in initiation of ACPA formation as antigen presenting cells. Gemmel et al. (2003) described that a predominance of B cells was found in moderate and extensive infiltrates of periodontal lesions, which can be regarded as a measure for inflammation. In our study the mean number of B cells was also increased in citrulline positive cells, however not significantly raised, probably due to small numbers.

No significant differences were found with regards to any of the

other investigated parameters between citrullinated protein positive and negative periodontitis stroma (i.e. age, severity of periodontitis, gender, and smoking). However this may be due to the small number of patients.

The question might rise why the buccal mucosa cells we used as a positive control in our study stained positive for both F95 and Ab5612. This might be due to the way the buccal cells were collected, i.e. by a swab technique and not by an incision biopsy. When applying a swab technique, the apoptotic loosely

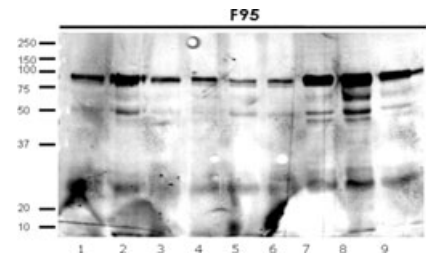


Fig. 6. Citrullinated proteins in periodontal tissue and synovial tissue detected by Western blotting with F95. Lane 1: periodontal tissue from RA patient with periodontitis, lanes 2-6: periodontal tissue from patients with periodontitis, lanes 7-9: synovial tissue from RA patients.

attached buccal mucosa cells are collected and it is known that such cells stain positive for antiperinuclear factor (Brouwer et al. 2006, Gyorgy et al. 2006). Thus, the presence of citrullinated proteins targeted by Ab5612 and F95 in buccal mucosal cells of healthy donors is most likely physiological and unrelated to ACPA associated aetiology of RA.

Although this study is the first to show citrullinated protein presence in periodontitis tissue, ACPA presence and local ACPA formation in periodontitis tissue and peripheral blood were not assessed. Neither was *P. gingivalis* presence assessed. It is not possible to stain for PPAD, as no antibodies are available to this antigen and staining for human PAD4 showed positive neutrophils in the tissue (data not shown). Thus, the most important focus of future studies should be on potential ACPA formation within periodontitis tissue. We are currently investigating presence of ACPA in serum of patients with severe periodontitis. Other factors related to ACPA formation in periodontitis should also be assessed in studies with a larger sample size, i.e. smoking habits, the types of citrullinated proteins present as well as the specific role of *P. gingivalis*, the presence of HLA DR B1 shared epitope alleles and the role of auto-antibody producing plasma cells and collagen auto-immunity.

In conclusion, citrullinated protein formation within the periodontal stroma appears to be an inflammation-dependent process. In periodontal epithelium, inflammation may lead to the formation of different types of citrullinated proteins



(targeted by F95) in addition to those naturally present in non-inflamed periodontal epithelium (targeted by Ab5612). Citrullinated proteins formed in periodontitis tissue appear to be of a similar variety as RA-affected synovial tissue. This may be due to the fact that periodontitis is a chronic inflammation of tissue containing many of the structural components of joints, including collagen, and all the major auto-antigens targeted by ACPA (Fig. 1). Moreover, periodontitis is associated with auto-antibody producing B-cells. Thus, in the presence of HLA-DR B1 shared epitope and exposure to tobacco smoke, periodontitis is postulated to provide an environment capable of the initiation of an ACPA response that might specifically target citrullinated proteins in joints.

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**Clinical Relevance**

*Scientific rationale for the study:* Anti-citrullinated protein antibodies (ACPAs) may play a role in the aetiology of rheumatoid arthritis (RA). Periodontitis, contains all of the ingredients necessary to induce ACPA formation. However,

whether citrullinated proteins are present in periodontitis tissue currently remains unknown.

*Principal findings:* Periodontitis leads to the formation of additional types of citrullinated proteins which appear to be similar to those formed in RA-affected synovial tissue.

*Practical implications:* In the presence of a certain genetic predisposition and smoking, citrullinated proteins formed in association with periodontitis may play a role in ACPA formation. Periodontitis might contribute to RA development.